

Malignant Transformation of Immortalized Human Bronchial Epithelial Cells by Asbestos Fibers

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Although asbestos is a well-established lung carcinogen, there currently is no suitable human cell model in which to examine the underlying cellular and molecular changes associated with fiber-mediated bronchial carcinogenesis. Using a recently established transformation model based on a human papillomavirus-immortalized human bronchial epithelial cell line, we successfully transformed these BEP2D cells after a single, 7-day treatment with a 20- $\mu\text{g}/\text{ml}$ (4 μg per cm^2 area) dose of Union Internationale Contre le Cancer (UICC) Rhodesian chrysotile fibers. Asbestos treatment resulted in a surviving fraction of 0.18 compared to control cells. Transformed cells developed through a series of sequential steps, including altered growth kinetics, resistance to serum-induced terminal differentiation, and anchorage-independent growth, before becoming tumorigenic to form progressively growing tumors in nude mice. Seven tumorigenic cell lines were isolated and determined to be of human epithelial origin based on immunofluorescent staining of keratin and isozyme analysis. Analysis of tumor DNA revealed no mutations at either codon 12 or 13 in any the *ras* oncogenes. An independent role for K-*ras* mutation in fiber carcinogenesis, therefore, cannot be confirmed. This model provides a unique opportunity to study the cellular and molecular changes at the various stages in fiber-mediated neoplastic transformation of human bronchial epithelial cells. — *Environ Health Perspect* 105(Suppl 5): 1085–1088 (1997)

Key words: asbestos, bronchial epithelial cells, fibers, chrysotile, bronchial carcinogenesis, immunofluorescent staining

Introduction

Although asbestos is carcinogenic and induces both bronchogenic carcinoma and mesothelioma in man, the underlying mechanisms of fiber carcinogenesis are not entirely clear at present. Various *in vitro* and *in vivo* studies, however, have suggested that fiber dimensions, surface properties, and physical durability are important criteria for the carcinogenicity of the fibers (1,2). Studies using oncogenic transformation as

an end point have shown that asbestos fibers can induce morphologically transformed foci in certain rodent cells (3). In addition, a supra-additive oncogenic transformation incidence has been demonstrated in rodent cells treated with asbestos fibers in combination with either ionizing radiation or chemical carcinogen [for review, see Hei (4)]. Recent evidence suggests that oxygen radicals may be important mediators in the toxicity, oncogenic transforming potential, and genotoxicity of asbestos fibers (2). Several studies have shown that the iron present in many types of carcinogenic fibers provides the necessary catalyst in the formation of reactive oxygen species through a series of one-electron reductions of molecular oxygen (5,6). The mutagenic effects of asbestos in mammalian cells have recently been demonstrated using a model system that can detect gross chromosomal deletions (4,7). These findings provide the first direct link between chromosomal abnormalities that frequently occur in fiber-exposed human and rodent cell lines and carcinogenicity

in vivo. The observation that antioxidant enzymes such as catalase and superoxide dismutase protect cells against the mutagenic effects of asbestos provides further evidence for the role of oxyradicals in fiber toxicology (8).

For a better understanding of the cellular and molecular mechanisms involved in human bronchial carcinogenesis by asbestos fibers, it would be ideal to use a human bronchial cell line that has been exposed to asbestos to assess the various transformation stages leading to malignancies. Until recently, no human cell model has been available for these studies because human cells in culture are refractory to malignant transformation *in vitro* (9). Recent studies using a human papillomavirus-immortalized human bronchial epithelial cell line (BEP2D) have shown that malignant transformation is induced by treatment with a single, 30-cGy dose of radon alpha particles at a frequency estimated to be approximately 4×10^{-7} after successive cultivation for 3 to 4 months postirradiation (10,11). In the present studies, we show that a single, 7-day treatment with chrysotile fibers induces malignant conversion of these immortalized cells. Transformed cells progress through sequential stages, including altered growth kinetics, resistance to serum-induced terminal differentiation, and anchorage-independent growth, before becoming tumorigenic and producing progressively growing subcutaneous tumors upon inoculation into athymic nude mice.

Materials and Methods

Cell Culture

The human papillomavirus (HPV18) immortalized human bronchial epithelial cell line at passages 21 and 22 was used in these studies (12). These cells have near diploid karyotype and have been in culture for more than 200 population doublings. They are anchorage dependent and non-tumorigenic in nude mice. Cells were routinely cultured in serum-free LHC-8 medium (Biofluid, Rockville, MD) supplemented with epidermal growth factor and other growth supplements as described previously (10–12). Parental and tumorigenic BEP2D cells were negative for *Mycoplasma* contamination as determined by either DNA fluorochrome staining or polymerase chain reaction (PCR) amplification with the *Mycoplasma* detection kit (American Type Culture Collection, Rockville, MD).

This paper is based on a presentation at The Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at EHP 26 March 1997; accepted 1 July 1997.

This work was supported in part by grants ES05786 and ES06831 from the National Institute of Environmental Health Sciences.

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Abbreviations used: PCR, polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol acetate; UICC, Union Internationale Contre le Cancer.

Asbestos Treatment

Union Internationale Contre le Cancer (UICC) standard reference Rhodesian chrysotile fibers (average length, 7.8 μ ; average diameter, 0.2 μ) were used in the study. The compositional analysis, size distribution, and preparation of the fibers were described previously (4,7). Briefly, chrysotile fibers were weighed, suspended in distilled water, autoclaved to sterilize, and used at the concentration indicated. The fibers were dispersed by sonication for 5 min before being diluted with tissue culture medium for cell treatment. Exponentially growing BEP2D cells ranging in number from 3×10^6 to 5×10^6 cells were treated with either a 10 or 20 μ g/ml dose of fibers for a period of 7 days. After treatment, cells were trypsinized and resuspended in serum-free medium. Aliquots were then plated into 60-mm diameter dishes to determine clonogenic survival and the remaining cells were replated for transformation assays as described previously (10,11).

Assay for Transformed Phenotypes

Alteration in growth kinetics, resistance to serum-induced terminal differentiation, anchorage-independent growth, and tumorigenicity in nude mice were assayed as described previously (10,11). The epithelial origins of BEP2D cells and their transformed variants were determined by immunohistochemical staining of keratin. Briefly, exponentially growing BEP2D cells on glass chamber slides (Nunc, Inc., Naperville, IL) were washed and stained with mouse antihuman cytokeratin CK5

(ICN Immunobiological, Lisle, IL) for 60 min at 37°C. The slides were then rinsed and counterstained with goat antimouse IgG-FITC (Sigma Chemical Co, St. Louis, MO) for 15 min at room temperature. After washing, the slides were mounted in buffered glycerol and examined under a fluorescent microscope. To establish cultures from either the primary or secondary tumors, nodules were resected under aseptic conditions, minced into 1-mm² fragments, and subjected to 0.1% collagenase treatment (Sigma, Type IV) for 2 hr at 37°C as previously described (10). After centrifugation at 200 \times g for 5 min, the resulting pellets were trypsinized for 4 min with 0.05% trypsin-EDTA solution (GIBCO, Grand Island, NY). The cell suspension was then centrifuged again before resuspending in medium and plating out in culture dishes. The viability of cell suspension determined by trypan blue exclusion assay was consistently more than 70%.

Detection of *ras* Mutation Using Polymerase Chain Reaction Amplification and Restriction Enzyme Analysis

Genomic DNA was amplified using *ras*-specific oligonucleotides that were slightly altered from the genomic sequences to create primer-mediated restriction sites that overlapped *ras* codons (13). A mutation at the respective position was associated with the loss of a restriction enzyme site, except for the aspartic acid mutation at codon 13 of the K-*ras*, which resulted in the gain of a restriction site. High molecular weight DNA from the various tumorigenic and transformed cell lines were prepared using standard phenol chloroform extraction, as described previously (7,10). The 5' primer used was ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT, and the 3' primer sequence was TCA AAG AAT GGT CCT GGA CC. For codon 12, the endonuclease *Bst*N1 was

used, whereas *Hph*1 was used for codon 13 as previously described (11,13).

Results

BEP2D cells grow in culture as a contact-inhibited monolayer with an epithelial morphology. At confluency, they have a saturation density of approximately 9.5×10^4 cells/cm² of growth area. The plating efficiency of the cell is between 15 and 25%, with an average population doubling time of approximately 35 hr when cultured in serum-free LHC medium (10,11). Chrysotile asbestos induced a dose-dependent decrease in the clonogenic survival of BEP2D cells (Figure 1). A single treatment with a 20- μ g/ml dose of fibers for a period of 7 days resulted in a survival fraction of approximately 18% compared to control cells. When fiber-exposed BEP2D cells were subcultured and injected into nude mice at this early stage, no tumor formation was observed (Asb7Fc) (Table 1). It was only with extensive subcultivation and the accumulation of additional phenotypic/mutagenic changes that tumorigenicity in nude mice could be demonstrated.

Control cells stop cellular division and undergo terminal differentiation in the presence of serum and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Figure 2). In the presence of serum and TPA, the normal plating efficiency of BEP2D cells decreased to approximately 1% of the original value in serum-free medium. In contrast, transformed cells were much less responsive. There was no difference in the colony growth for the tumorigenic clone AsbTL cells whether or not serum and TPA were present. In contrast, the anchorage-independent, non-tumorigenic cell line, Asb7FA5, had a colony-forming efficiency that was approximately 70% of the control in the presence of both serum and TPA (data not shown). These data are consistent with the previous observation that malignant

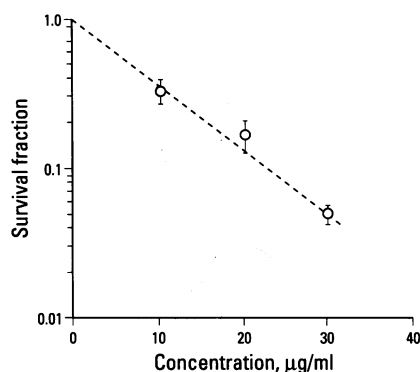


Figure 1. Effects of graded doses of chrysotile asbestos on clonogenic survival of BEP2D cells after a single, 7-day treatment. The fiber concentration can be converted to grams per square centimeter by dividing the dose in grams per milliliter by 5. Each data point represents pooled results from three experiments. Bars represent \pm SEM.

Table 1. Characterization of chrysotile asbestos-treated BEP2D cells.

Cell line	Dose, μ g/ml	Time in culture, weeks ^a	Growth in agar ^b	Tumorigenicity ^c	Cell line established ^d
Asb7Fc	20	2	—	0/3	—
Asb7	20	11	++	2/2	2
AsbTL	20	3	+++	3/3	5
BEP2D	0	>22	—	0/37	—

^aTime lapse between fiber treatment and testing for transformed phenotypes. ^bAnchorage-independent growth ranged from approximately 0.25 to 0.8%. ^cNumber of animals with tumor divided by the total number of animals injected. Each animal was injected at two different sites. A total of seven tumors were obtained from the five nude mice. No tumor was formed in the three control animals or of a total of 37 mice tested, including historical controls.

^dNumber of tumor cell lines established; isozyme analysis and keratin stain confirmed their human epithelial origin.

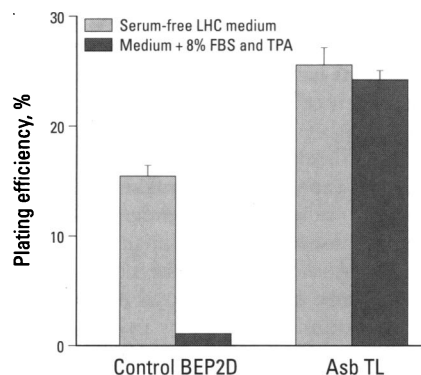


Figure 2. Clonal growth efficiency of control BEP2D cells and the fiber-induced tumorigenic AsbTL cells in either serum-free LHC medium or medium supplemented with 8% FBS and 100 ng/ml TPA (Midwest Research Institute, Kansas City, MO). Bars represent \pm SEM.

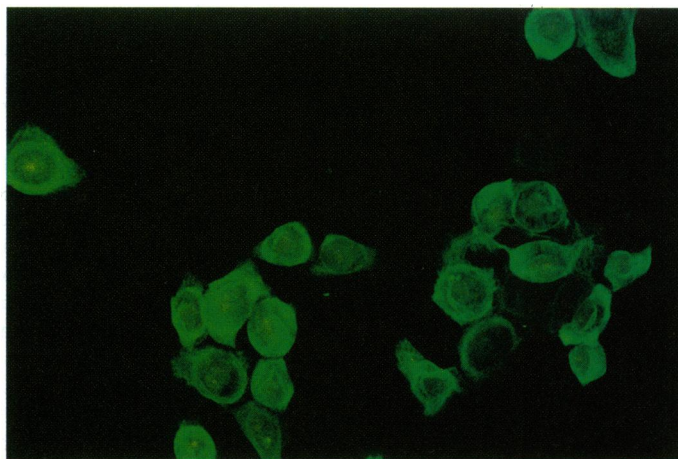


Figure 3. Representative immunofluorescent staining of human cytokeratin from AsbTL, a tumorigenic BEP2D cell induced by chrysotile fibers. Note that the nuclei were counterstained with methyl green (magnification, $\times 400$).

epithelial cells are resistant to serum factors that induce terminal differentiation in normal epithelium (10,14).

Transformed and tumorigenic BEP2D cells induced by chrysotile fibers were tightly packed in culture. Although they had similar growth kinetics when compared with control cells, they routinely showed a 2- to 3-fold higher saturation density than the latter (10). Table 1 listed the anchorage-independent and tumorigenic characteristic of control cells and two asbestos-induced tumorigenic cell lines. Exponentially growing bronchial epithelial cells treated with chrysotile fibers were subcultured continuously for 11 weeks before isolates of agar-positive colonies emerged at frequencies ranging from 0.25 to 0.8%. Upon inoculation into nude mice, these cells produced tumors with a latency period of 8 to 10 weeks at the site of injection. It should be noted that although most agar-positive BEP2D clones were nontumorigenic, they all demonstrated the propensity to resist serum-induced terminal differentiation. In contrast, the majority of tumorigenic BEP2D cells demonstrated anchorage-independent growth, which illustrated the progressive nature of the malignant phenotypes. When the tumors had grown to a diameter of 1 cm, they were excised. Routine hematoxylin and eosin staining of paraffin-embedded sections indicated that the tumors were carcinoma in nature.

Immunohistochemical staining for human cytokeratin indicated that the tumor cells were of epithelial origin. (Figure 3). The normal keratin expressed in BEP2D cells was also expressed in

various transformed stages. In contrast, normal fibroblasts that were used as negative controls in the staining procedure were negative for keratin expression. It should be noted that control cells subcultured under identical conditions did not produce any anchorage-independent clones. In addition, none of the three animals injected with the control cells together with 34 other historical controls produced tumors up to 22 weeks postinoculation. Overall, two primary tumorigenic cell lines were established (Table 1). One of these lines, AsbTL, was subsequently expanded in culture and reinoculated into nude mice. The secondary tumors developed within a shorter latency period of 2 to 3 weeks. These studies show that chrysotile-treated BEP2D cells undergo gradual, sequential changes of altered growth and anchorage-independent growth before becoming tumorigenic in nude mice. In addition, each preceding stage represents a necessary but insufficient step toward the later, more malignant phase.

Little information is available on the functional role of oncogenes and tumor-suppressor genes in fiber-mediated bronchial carcinogenesis. Recent evidence suggests that mutations in *K-ras* are frequent in lung tumor specimens from asbestos workers (15). However, most patients in these studies were also smokers. There is no evidence for the independent role of *K-ras* mutations in fiber carcinogenesis. Figure 4 shows the detection of *K-ras* codon 13 mutations among the various tumorigenic BEP2D cells induced by asbestos fibers using a PCR-based restriction enzyme-mediated assay. A mutation at

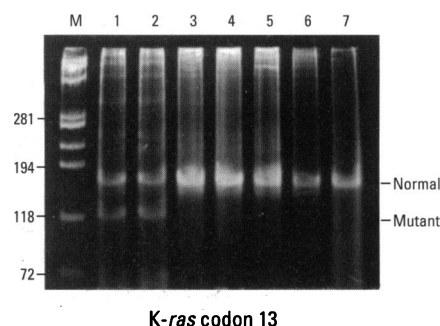


Figure 4. Detection of *K-ras* mutation at codon 13 using PCR amplification and restriction enzyme analysis. ϕ x 174 was used as a size marker (lane M). Lanes 1 and 2 are positive controls from HCT116 human colon carcinoma cell lines. Mutations at codon 13 create the recognition site for *Hph*I and cleave the fragments. Lanes 3–6 are four representative tumorigenic BEP2D cells induced by chrysotile fibers, and lane 7 is the control BEP2D cells.

codon 13 resulted in the replacement of glycine by aspartic acid and created the *Hph*I recognition sequence GGTGA. Normal fragments without the mutation remained undigested, as shown by control cells (Figure 4, lane 7), whereas DNA samples with mutations at codon 13, as shown by the positive control HCT116 human colon carcinoma cells, were digested (Figure 4, lanes 1 and 2). None of the four asbestos-induced tumorigenic cell lines examined demonstrated mutations at codon 13 of the *K-ras* (Figure 4, lanes 3–6). Similar negative results were obtained at codon 12 of *K-ras* and at codons 12 and 13 of *H-ras* and *N-ras* (data not shown).

Discussion

The mechanisms of fiber carcinogenesis are poorly understood. Asbestos induces chromosomal aberrations, sister chromatid exchanges, and multilocus deletions in mammalian cells (2,7,8). In addition, the human chromosomes frequently found to be deleted in asbestos-associated mesotheliomas, such as chromosomes 3p, 9p, 11p, 13q, and 22q (16,17), are often associated with genes with known tumor-suppressor functions (17,18). The availability of a human cell transformation model based on bronchial epithelium in which the malignant stage develops through a series of sequential steps will provide a unique opportunity to study the cellular and molecular changes associated with fiber-mediated carcinogenesis.

BEP2D cells constitute a suitable model for the study of the various stages of fiber-induced human bronchial carcinogenesis. These cells were initiated by lipofectin

transfection of cloned full-length HPV18 into normal human bronchial epithelial cells obtained as outgrowths of bronchial explants (12). Although these bronchial epithelial cells are immortal, they are genotypically stable and express human cytokeratin (10,11). They are anchorage dependent and do not form tumors in immunosuppressed host animals. The observation that HPV-immortalized BEP2D cells are nontumorigenic even in late passages suggests that alterations in *p53* and *Rb* functions are not sufficient criteria for tumorigenic development and that additional genetic changes are needed. The finding that mutations in the *Rb* or *p53* genes are not rate limiting for asbestos-induced mesotheliomas (19) provides further support for the suitability of BEP2D cells in the study of the molecular mechanisms of fiber carcinogenesis.

Molecular epidemiologic studies have shown that the incidence of K-*ras* mutations

ranged from 30 to 60% among human lung cancers. The highest incidence of such mutations has been reported by Husgafvel-Pursiainen et al. (15) in 60% of the cases among asbestos miners, which indicates a strong correlation between smoking and asbestos exposure. Unfortunately, there is no evidence for the independent role of K-*ras* mutations in fiber carcinogenesis. In the present studies, none of the fiber-induced tumorigenic cell lines showed mutations at either codon 12 or 13 in any of the *ras* oncogenes. These results are consistent with our previous findings that most asbestos-induced mutants are multilocus deletions rather than point mutations (7,8). Our results suggest that chemical carcinogens in tobacco smoke such as benzo[*a*]pyrene and the tobacco-specific nitrosamines may be the likely inducers of K-*ras* mutations among samples of adenocarcinomas from asbestos miners.

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